Studies on the pathogenesis of avian influenza in chickens

(ニワトリのインフルエンザ病態発現機序に関する研究)

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Contents

Abbreviations

CBB	Coomassie Brilliant Blue
CHO	Chinese hamster ovary cell
CIPR	Cold-inducible RNA binding protein
EID_{50}	50% egg infectious dose
HA	hemagglutinin
HI	hemagglutinin-inhibition
HPAIV	highly pathogenic avian influenza virus
IL	Interleukin
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IVPI	Intravenous Pathogenicity Index
LPAIV	low pathogenic avian influenza virus
NA	neuraminidase
PBS	phosphatase buffered saline
rchIL-6	recombinant chicken IL-6
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
STAT	signal transducer and activator of transcription

TF	Tissue factor
TNF-α	Tumor necrosis factor alpha
WB	western blotting

Preface

Influenza A virus infections are found in a variety of birds and mammals including humans. These viruses are classified into subtypes on the basis of the antigenic specificity of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. To date, viruses of 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) have been isolated from avian species [2, 12]. Influenza A viruses infecting chickens are categorized into two pathotypes based on their virulence to chickens; namely, low pathogenic avian influenza virus (LPAIV) and highly pathogenic avian influenza virus (HPAIV). The HA of avian influenza virus is a primary viral protein to determine the pathogenicity in chickens. The HAs of HPAIVs have at least a pair of di-basic amino acid residues at their cleavage site, which permits cleavage activation by ubiquitous proteases such as furin and PC6, leading to systemic infection in chickens [17, 54]. On the other hand, the HAs of LPAIVs are cleaved only by trypsin-like proteases expressed in the respiratory and intestinal tracts, causing local infection in chickens. Highly pathogenic avian influenza caused by HPAIV has occurred worldwide and led to serious economic losses in poultry industry. The sporadic infections with H5 or H7 HPAIV in humans have been also reported and 648 people were infected with H5N1 HPAIV and 384 deaths were confirmed for the last decade [66].

The pathogenicity of infectious agents is determined by the host-pathogen interactions. Innate immune system is one of the host factors to interact with pathogens and it is the first line of the defense against infection. Production of cytokines and chemokines in the innate immune system is critical response to induce inflammation and to eliminate pathogens in the early stage of infection. High levels of cytokines and chemokines were found in severe cases of H5N1 influenza virus infection in birds and mammals [4, 10, 19, 26, 55, 64]. Therefore, aberrant cytokine response called “cytokine storm” is hypothesized to be the main cause of high mortality in HPAIV infection in animals. However, H5 HPAIV infection was also lethal to mice genetically lacking cytokines or chemokines such as Tumor necrosis factor alpha (TNF-α), Interleukin (IL)-1, IL-6, and CC chemokine ligand 2 or those receptors, and immunosuppressive treatment was not always an effective therapy for H5 HPAIV infection [8, 46, 50]. It has been still controversial whether aberrant cytokine response is the cause or effect of severity of influenza in birds and mammals.

In Chapter I, it is described that virus growth and cytokine response in chickens experimentally infected with two H7 HPAIVs and an H5 LPAIV were analyzed to examine the relationship between pathogenicity of avian influenza viruses and host responses. In Chapter II, it is described that the role of extensive response of cytokines, especially IL-6, in the pathogenesis of HPAIV infection in chickens was investigated

using recombinant chicken IL-6 (rchIL-6) and anti-rchIL-6 antibodies. The findings obtained in the present studies should contribute to the better understanding of the pathogenesis of highly pathogenic avian influenza in chickens and the development of therapeutic drugs for proper treatment of severe cases of influenza in humans.

Chapter I

Pathogenicity of highly pathogenic avian influenza virus in chickens

Introduction

HPAIVs cause lethal systemic infection in chickens. It is known that the clinical course of the infection with HPAIVs depends on virus strains. Chickens infected with HPAIVs generally show ruffled feathers, depression, and edema of the face, comb, and wattles; they develop subcutaneous hemorrhages in unfeathered skin and die within a few days [57]. In some acute cases of the disease, chickens infected with HPAIV suddenly die without showing apparent clinical signs or gross lesions [35, 36, 56]. In most cases, high titers of viruses were recovered systemically and hemorrhagic or necrotic foci were found in the tissues of dead chickens [57]. Although the HA is the primary factor for systemic proliferation of HPAIVs in chickens, it is assumed that host factors are involved in the sudden death of the chickens.

Cytokines are regulators in many biological processes including cell growth, differentiation, and survival, and including hematopoiesis, inflammation, and immunity

to infection. In the course of infection, the balance of pro-inflammatory and anti-inflammatory cytokines is important for induction of a proper immune response, clearance of pathogen, and healing. In particular, pro-inflammatory cytokines such as IL-1β, IL-6, and Interferon (IFN)-γ mediate rubor, fever, pain, edema, and cellular infiltration [20]. Excessive cytokine responses have been found in cases of lethal infection with HPAIVs in humans, mice, ferrets, and monkeys [3, 5, 10, 26, 62]. As with the cases in mammals, high expression of mRNA of cytokines such as IL-6, IL-12, and IL-18 was observed in the lungs and spleen of the chickens infected with H5 HPAIV, and a large amount of type I IFN was also detected in the tissues and plasma of the chickens infected with H5 HPAIV [19, 31, 45, 55]. In contrast, some strains of H5 or H7 HPAIVs did not induce significant cytokine mRNA expression in chicken lungs in the early stage of infection [48, 55]. The relationship in the outcomes of HPAIV infection and cytokine response has not been well understood in chickens.

In order to examine the relationship between pathogenicity of HPAIV and host cytokine response during the early stage of infection, two H7 HPAIVs and a LPAIV were intranasally inoculated into chickens, and viral proliferation, mRNA expression of cytokines, and capillary permeability were analyzed.

Materials and Methods

Viruses

The two HPAIVs A/turkey/Italy/4580/1999 (H7N1) (Ty/Italy) [7] and A/chicken/Netherlands/2586/2003 (H7N7) (Ck/NL) [22] were kindly provided by Dr. I. Capua, Instituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Padova, Italy). The intravenous pathogenicity index (IVPI) and the 50% chicken lethal dose of Ty/Italy after intranasal inoculation were 3.00 and $10^{2.0}$ 50% egg infectious dose (EID_{50}), whereas those of Ck/NL were 2.68 and $10^{5.8}$ EID_{50}, respectively. The LPAIV A/chicken/Ibaraki/1/2005 (H5N2) (Ck/Ibaraki) [41] was kindly provided by Dr. S. Yamaguchi, National Institute of Animal Health (Tsukuba, Ibaraki, Japan), and the IVPI was 0.00 [40]. Viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 40–48 h.

Experimental infection of chickens with influenza viruses

In brief, $10^{6.0}$ EID_{50} of Ty/Italy or Ck/NL was intranasally inoculated into eight 4-week-old chickens (Boris brown) (Hokuren, Hokkaido, Japan). The chickens were observed every day until 12 days post inoculation (dpi). Sera from the surviving chickens were examined using a hemagglutination–inhibition (HI) test [51] at 12 dpi.

To examine the proliferation of each virus and the host cytokine response during the early stage of infection, $10^{6.0}$ EID_{50} of Ty/Italy, Ck/NL, or Ck/Ibaraki was each intranasally inoculated into groups of twelve 4-week-old chickens (Boris brown and Juria) (Hokkaido Chuo Shukeijo Corporation, Hokkaido, Japan). Three chickens per group were euthanized, and the brains, lungs, and spleens were collected at 24, 48, 72, and 96 hours post inoculation (hpi). At 48 and 96 hpi, the tissue specimens were soaked in 10% formalin for histopathological analysis. To determine virus infectivity titers, the tissues were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and 10% (w/v) suspensions were made with minimum essential medium (Nissui, Tokyo, Japan) containing 100,000 U/ml penicillin (Meiji Seika, Tokyo, Japan), 10 mg/ml streptomycin (Meiji Seika), 0.3 mg/ml gentamicin (Merk, New Jersey, U.S.A.), 0.2% nystatin (Life Technologies, California, U.S.A.), and 0.5% bovine serum albumin fraction V (Roche Diagnostics, Mannheim, Germany). These suspensions and peripheral blood were serially diluted with PBS and inoculated into 10-day-old embryonated chicken eggs, and incubated at 35°C for 48 h. Virus titers were determined according to the Reed and Munch method [49] and were expressed as EID_{50} per gram of tissue or milliliter of blood. For quantitative real-time PCR analysis of the mRNA expression of cytokines, a portion of each tissue was soaked in RNAlater (Ambion, Texas, U.S.A.) and stored at −20°C.

All animals were housed in self-contained units (Tokiwa Kagaku, Tokyo, Japan) in the BSL-3 facility of the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The institutional animal care and use committee of the Graduate School of Veterinary Medicine approved these animal experiments (approval numbers: 09-1051, 11-1112), and all experiments were performed under the guidance of the Institute for Laboratory Animal Research.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from each tissue using the RNeasy Mini Kit (QIAGEN, Maryland, U.S.A.) according to the manufacturer's instructions. To remove genomic DNA, total RNA was treated with DNase I (QIAGEN). One microgram of total RNA per sample was reverse-transcribed with Oligo $(dT)_{20}$ primers, RNase inhibitor (Life Technologies, California, U.S.A.), and M-MLV reverse transcriptase (Life Technologies). The reaction mixtures comprised 2 μl of cDNA, 10 μl of Light Cycler 480 SYBR Green I master mix (Roche Diagnostic) or KAPA SYBR Fast qPCR master mix (KAPA, Boston, U.S.A.), 2 μl of forward and reverse primer (10 μM), and 4 μl of pure water. Reactions were carried out on a Light Cycler 480 System II (Roche Diagnostic). The primers were as follows: β-actin (forward: 5′-CTG TTC GCC TTT CAG ACC TAC A-3′, reverse: 5′-CAT GGT GAT TTT CTC TAT CCA GTC C-3′)

(Accession number: NM_205518), IFN-α [24], TNF-α [37], IFN-γ, IL-1β, and IL-6 [18]. The copy number of cytokine mRNA was normalized to that of β-actin, and the data was shown as mean fold change compared with that of uninfected control birds.

Immunohistochemistry and in situ hybridization

Tissues were fixed with 10% formalin and embedded in paraffin and sectioned at 3–4 μm. For light microscopy, the sections were subjected to hematoxylin–eosin staining. To detect viral antigens, the sections were stained using the streptavidin–biotin–immunoperoxidase complex method with the Histofine SAB-PO (M) kit (Nichirei Biosciences, Tokyo, Japan) according to the manufacturer's instructions. The sections were deparaffinized and digested with 0.1% trypsin at 37°C for 30 min, and endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol. After blocking of nonspecific reactions with normal goat serum, the sections were incubated with mouse anti-NP monoclonal antibody (produced in our laboratory; 1: 1,000) at 4°C for 12 h. The chromogenic reaction was carried out by incubating the sections in 0.05 M Tris-HCl buffer containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan), 0.005% H_2O_2, and 0.01 M imidazol (Life Technologies, California, U.S.A.), and the sections were counterstained with Mayer's hematoxylin (Wako, Osaka, Japan).

To detect IL-6 mRNA, *in situ* hybridization was performed using a QuantiGene viewRNA Tissue Assay (Affymetrix, California, U.S.A.). The viewRNA probe set consisted of 18 probes designed to cover 1,142 base pairs of avian IL-6 mRNA sequence (Accession number: MN_204628). In brief, after deparaffinization, brain sections from infected and uninfected chickens were treated with a target retrieval solution (DAKO, California, U.S.A.) at 95°C for 40 min, and with 0.1 μg/ml of proteinase K (DAKO) at 37°C for 15 min, as described previously [33]. They were subsequently incubated with a viewRNA probe at 40°C for 2 h. After washing 3 times in wash buffer, hybridization with PreAmplifier Mix QT (Affymetrix), Amplifier Mix QT (Affymetrix), and Label Probe 1 conjugated with alkaline phosphatase (Affymetrix) was performed according to the manufacturer's instructions. After incubation of FastRed substrate (Warp Red Chromogen Kit, Biocare Medical, California, U.S.A.), the slides were counterstained with Gill's hematoxylin (Wako).

Evaluation of the integrity of blood tissue barriers

Capillary permeability was evaluated by extravasation with Evans blue (Wako), as described previously [58]. The infected chickens and normal chickens (n = 5) were intravenously injected with 22 mg/kg of 2% Evans blue dye in sterile saline at 4 days after infection with Ty/Italy (n = 6), Ck/NL (n = 5), or Ck/Ibaraki (n = 5). After 3 h,

the chickens were exsanguinated, and brains, lungs, hearts, spleens, kidneys, and colons were collected. Portions of the tissues were soaked in 500 μl of formamide (Sigma Aldrich Japan, Tokyo, Japan) at 38°C for 24 h. The amount of Evans blue in supernatants was measured against a standard of 90% formamide in saline at 630 nm using a Model 680 Microplate Reader (Bio-Rad Laboratories, California, U.S.A.), and Evans blue concentrations (ng/g) were calculated for each tissue using a standard curve.

Statistical analysis

Statistical analyses of the results were made using unpaired, parametric or non-parametric Student's t-test. Differences were considered statistically significant when $P < 0.05$.

Results

Pathogenicity of HPAIVs in chickens

To compare the pathogenicity of Ty/Italy and Ck/NL in chickens, $10^{6.0}$ EID_{50} of each virus was inoculated intranasally into chickens. All the chickens inoculated with Ty/Italy showed lethargy, inner hemorrhage of the skin, edema of the face and legs, and red conjunctiva from 2–3 dpi. These symptoms worsened rapidly, and all chickens died by 4 dpi (Fig. 1). On the other hand, in the chickens infected with Ck/NL, symptoms appeared at 3–4 dpi and half of the chickens died at 6–7 dpi; and the others survived for the 12 observation days. HI antibodies against homologous virus antigens were detected in the sera of the surviving chickens at titers of 512–1,024.

Proliferation of HPAIVs and LPAIV in chickens

To compare the proliferation of Ty/Italy, Ck/NL, and Ck/Ibaraki in the chickens, infectivity titers in the peripheral blood, brains, lungs, and spleens of the chickens inoculated with each of the 3 viruses were determined at 24, 48, 72, and 96 hpi (Figs. 2A–2D). The Ty/Italy strain rapidly replicated in all tissues examined, and the highest infectivity titers were $10^{7.0}$ to $10^{8.0}$ EID_{50}/g in the brain at 72–96 hpi.

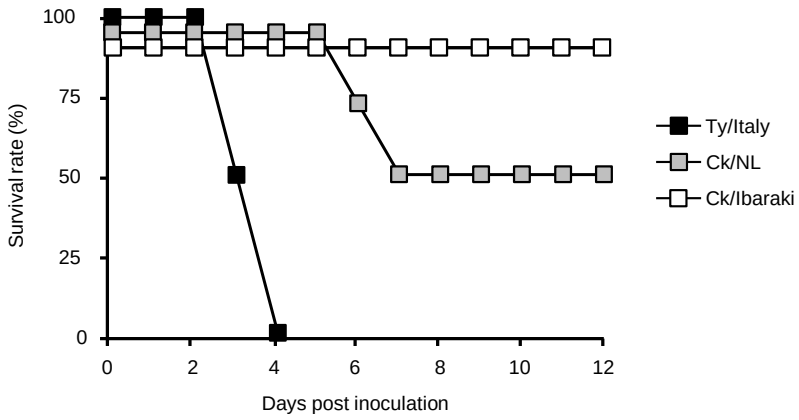

Fig. 1 Survival rates of the chickens inoculated with Ty/Italy, Ck/NL, and Ck/Ibaraki.
Chickens were intranasally inoculated with $10^{6.0}$ EID_{50} of Ty/Italy (n = 4) or Ck/NL (n = 4) and were observed for 12 days. Data of the chickens inoculated with Ck/Ibaraki was refered from Okamatsu *et al.* [35].

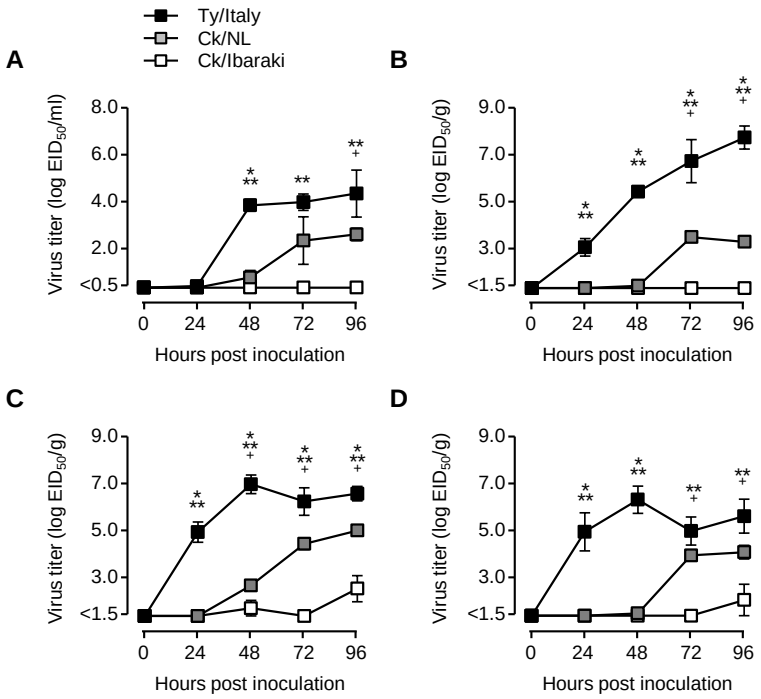

Fig. 2 Comparison of HPAIVs and LPAIV proliferation.

After $10^{6.0}$ EID_{50} of Ty/Italy, Ck/NL, or Ck/Ibaraki was intranasally inoculated into chickens, peripheral blood (A), brains (B), lungs (C), and spleens (D) were collected every 24 h, and infectivity titers were determined by inoculation of 10-day-old embryonated eggs. The mean values with corresponding standard errors from 3 chickens are shown. *, $P<0.05$ between Ty/Italy and Ck/NL; **, $P<0.05$ between Ty/Italy and Ck/Ibaraki; +, $P<0.05$ between Ck/NL and Ck/Ibaraki.

In contrast, Ck/NL replicated more slowly than Ty/Italy in all the chicken tissues, with infectivity titers 10^2–10^4 times lower than those of Ty/Italy. Infectious viruses in the tissues of the chickens inoculated with Ck/NL gradually decreased at 120 and 144 hpi (data not shown). Disease signs appeared in the chickens inoculated with Ty/Italy at 48 hpi, and one bird died at 2 dpi and another at 3 dpi. The chickens inoculated with Ck/NL showed mild disease signs after 72 hpi. On the other hand, no chicken inoculated with Ck/Ibaraki showed any disease signs, and viruses were slightly recovered only from their lungs (Fig. 2C) and spleens (Fig. 2D) at 48 and 96 hpi ($\leqq 10^{1.8}$ to $10^{3.7}$ EID_{50}). At 120 and 144 hpi, viruses were not recovered from the chickens inoculated with Ck/Ibaraki (data not shown). These three avian influenza viruses showed different patterns of virulence, and severe disease signs were accompanied by rapid viral proliferation in the chickens.

Cytokine response in the chickens inoculated with HPAIVs and LPAIV

To examine cytokine response to infection with each virus, the mRNA expression of the inflammatory cytokines IFN-γ, IL-1β, IL-6, and TNF-α and the antiviral cytokine IFN-α was analyzed in the brains, lungs, and spleens of the chickens inoculated with Ty/Italy, Ck/NL, or Ck/Ibaraki, respectively (Figs. 3A–3C). Strong or moderate expression of cytokines was observed in the tissues of the chickens infected with

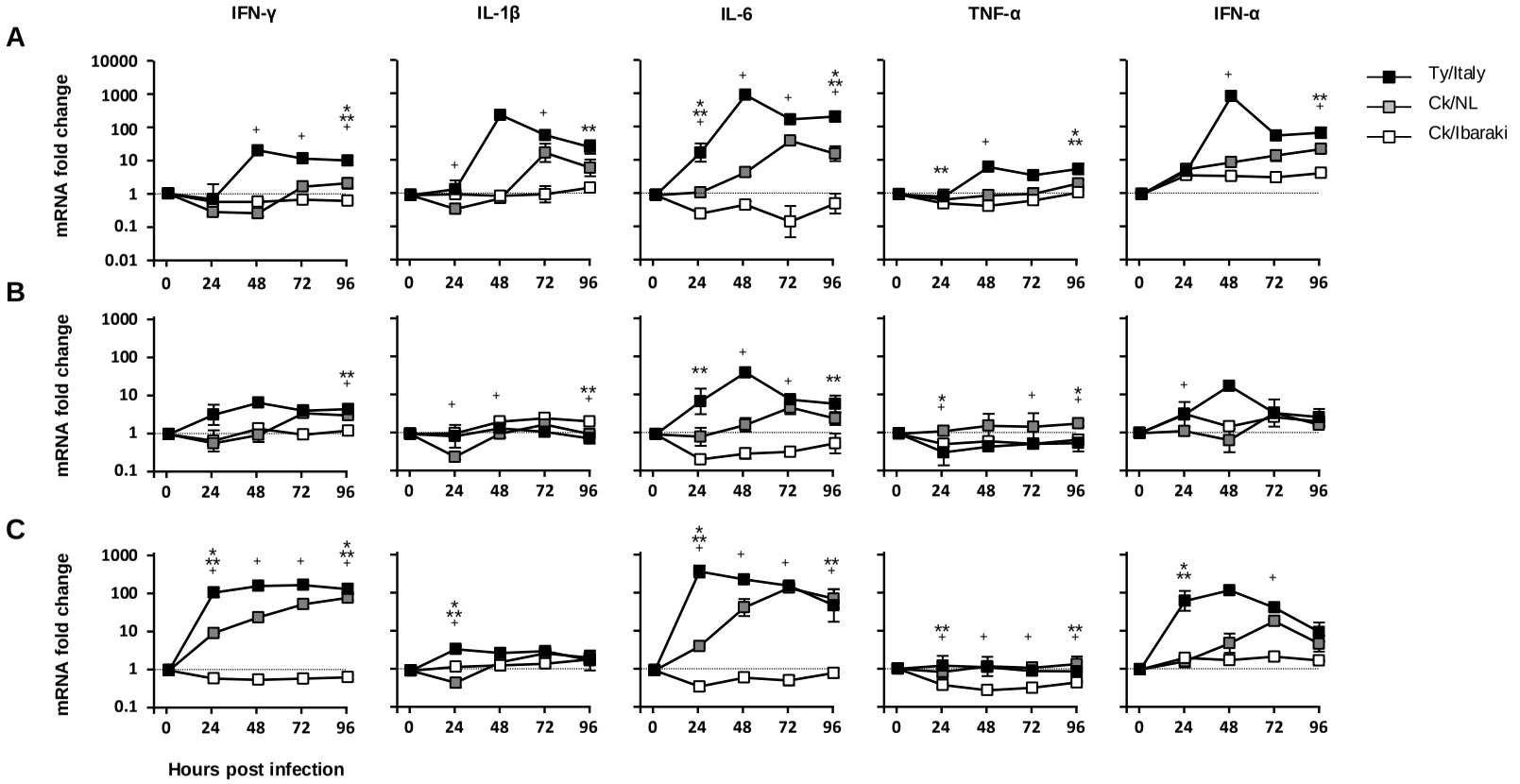

Fig. 3 Comparison of cytokine mRNA expression.

Tissues were collected from 3 chickens per group every 24 h after inoculation with $10^{6.0}$ EID_{50} of HPAIVs or LPAIV, and cytokine mRNA expression in the brain (A), lungs (B), and spleen (C) was analyzed using real-time PCR. Data are expressed as mean fold changes with standard errors relative to β-actin mRNA. Data of dead chickens was eliminated from the results. *, $P<0.05$ between Ty/Italy and Ck/NL; **, $P<0.05$ between Ty/Italy and Ck/Ibaraki; +, $P<0.05$ between Ck/NL and Ck/Ibaraki.

Ty/Italy or Ck/NL, respectively. In particular, cytokine expression was markedly elevated in the brains (Fig. 3A). The highest cytokine expression in the brains of the chickens inoculated with Ty/Italy was found at 48 hpi, with increase in IFN-γ, IL-1β, IL-6, TNF-α, and IFN-α levels by 21-, 286-, 1,179-, 7.0-, and 1,032-fold, respectively, compared to those of uninfected chickens. Cytokines were also strongly expressed in the brains at 96 hpi with Ty/Italy. In the brains of the chickens inoculated with Ck/NL, IL-1β, IL-6, and IFN-α levels were increased by 28-, 49-, and 25-fold, respectively, at 72–96 hpi. The levels of IFN-γ and TNF-α were not significantly changed after infection with Ck/NL. In the lungs (Fig. 3B) and spleens (Fig. 3C), the expression patterns of cytokines were similar to those in the brain. The largest increase in mRNA expression in the lungs of the chickens inoculated with Ty/Italy or Ck/NL was observed with 6.6- or 3.5-fold, 38- or 5.6-fold, and 16- or 6.3-fold increase in IFN-γ, IL-6, and IFN-α, respectively. In the spleens of the chickens infected with Ty/Italy or Ck/NL, IFN-γ, IL-6, and IFN-α levels were increased by 132- or 58-fold, 276- or 103-fold, and 98- or 15-fold, respectively. The increases of IL-1β and TNF-α levels were not apparent in the lungs and spleens of the chickens infected with Ty/Italy nor with Ck/NL. It is noteworthy that mRNA expression of IL-6 was most apparent among the 4 cytokines in each tissue of the chickens infected with HPAIVs. In contrast, the level of cytokine expression was not significantly high in each tissue of the chickens infected

with Ck/Ibaraki (≦3.5-fold). Thus, excessive cytokine responses were observed in the chickens infected with HPAIVs.

Distribution of virus antigen in the brain

To investigate the distribution of virus antigen in the brains of the virus-infected chickens, immunohistochemistry was performed on the brain sections at 48 and 96 hpi. The large amount of antigen was detected throughout the cerebrum of Ty/Italy-infected chickens at 96 hpi (Fig. 4a). Antigen-positive cells included neurons, ependymal cells, astrocytes, oligodendrocytes, microglia, endothelial, and necrotic cells (Figs. 4b–4d). In Ty/Italy-infected chickens, antigen was detected on the brain section at 48 hpi. In contrast, viral antigen-positive cells were not detected in the brains of the chickens infected with Ck/NL nor Ck/Ibaraki at 48 and 96 hpi (Figs. 4e and 4f).

Distribution of IL-6 mRNA in the brain

Based on real-time PCR analyses, the IL-6 response was most significant in the brains of the Ty/Italy-infected chickens. Subsequent *in situ* hybridization identified microglia as the main IL-6-producing cells in the brain, forming nodules or scattering in the parenchyma. Consistent with the results of mRNA expression, microglial nodules with IL-6 mRNA were frequently found in the sections from the Ty/Italy-infected

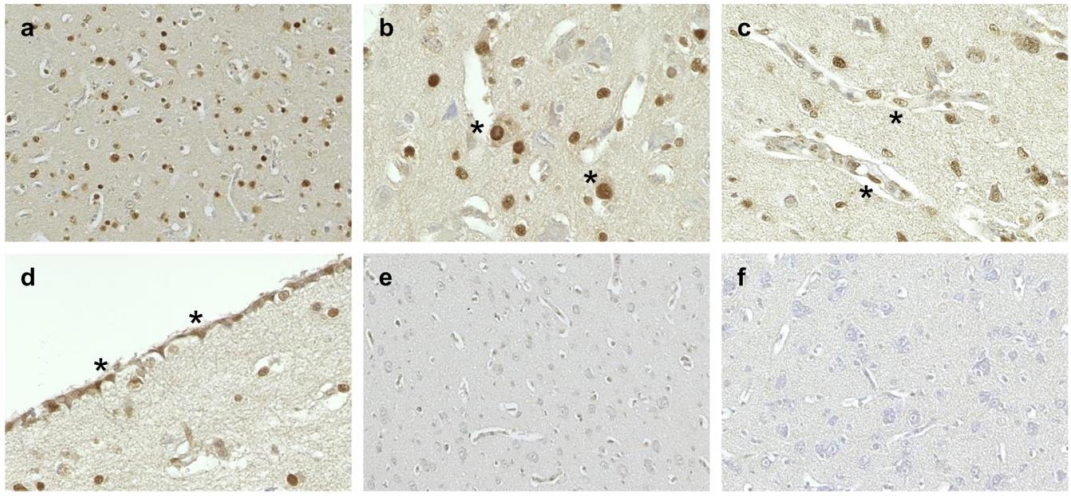

Fig. 4 Immunohistochemical analysis of viral antigens in the brain.

Viral antigen (brown signals) was detected in the Ty/Italy-infected chickens at 96 hpi (a). Antigen-positive cells included neurons (asterisks in b), endothelial cells (asterisks in c), and ependymal cells (asterisks in d). Virus antigen-positive cells were not detected in the brains of the Ck/NL- (e) nor Ck/Ibaraki- (f) infected chickens at 96 hpi. Original magnification, ×100: a, e, f; ×400: b, c, d

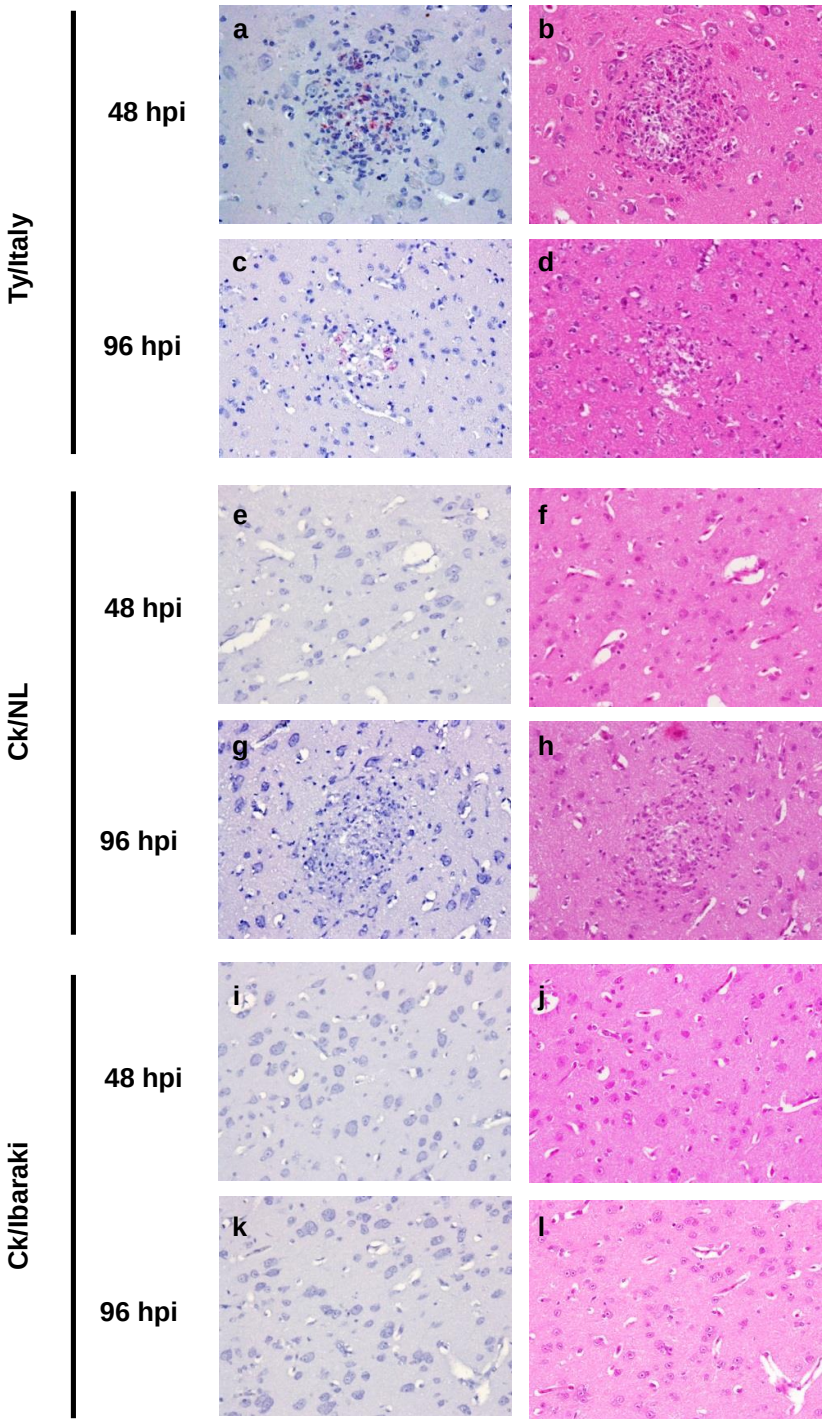

Fig. 5 Detection of IL-6 mRNA in the brain using *in situ* hybridization.

IL-6 mRNA expressing cells in the brains of the chickens infected with Ty/Italy were determined by *in situ* hybridization. IL-6 signals, represented as red color, were mainly localized to the microglial nodules on the section at 48 (a) and 96 hpi (c). The IL-6 mRNA was not detected in the brains of the Ck/NL-infected chickens (e and g), nor in those of the Ck/Ibaraki-infected chickens (i and k). The consecutive sections of hematoxylin eosin staining (b, d, f, h, j, and l) were examined by *in situ* hybridization (a, c, e, g, i, and k), respectively. Original magnification, ×400.

chickens obtained at 48 hpi (Fig. 5a), and were rarely found in these tissues at 96 hpi (Fig. 5c). On the brain section of Ck/NL-infected chickens, microglial nodules were found at 96 hpi lacking IL-6 mRNA signals (Fig. 5g). No microglial nodule or IL-6 mRNA positive cell was observed in the brain of the Ck/Ibaraki-infected chickens (Figs. 5i and 5k).

Extravasation of Evans blue in the tissues of the chickens inoculated with HPAIVs and LPAIV

To assess vascular permeability, Evans blue dye was intravenously injected into the chickens at 4 dpi with Ty/Italy, Ck/NL, or Ck/Ibaraki. The brains (Figs. 6A and 6B) and hearts (Fig. 6D) of the chickens infected with Ty/Italy turned blue, while those of the chickens infected with Ck/NL remained normal color. Blue spotted regions were particularly prevalent in three of the six birds infected with Ty/Italy. The Evans blue-stained hearts were found in two of the six chickens infected with Ty/Italy, while no staining was detected in the chickens inoculated with Ck/NL. Evans blue concentrations in the brains, hearts, spleens, kidneys, and colons of the chickens infected with Ty/Italy were significantly higher than those in the birds infected with Ck/NL ($P < 0.05$; Fig. 7). Based on macroscopic observation, the spotted region in the brain of the chicken infected with Ty/Italy was identified as the choroid plexus (Fig. 6B)

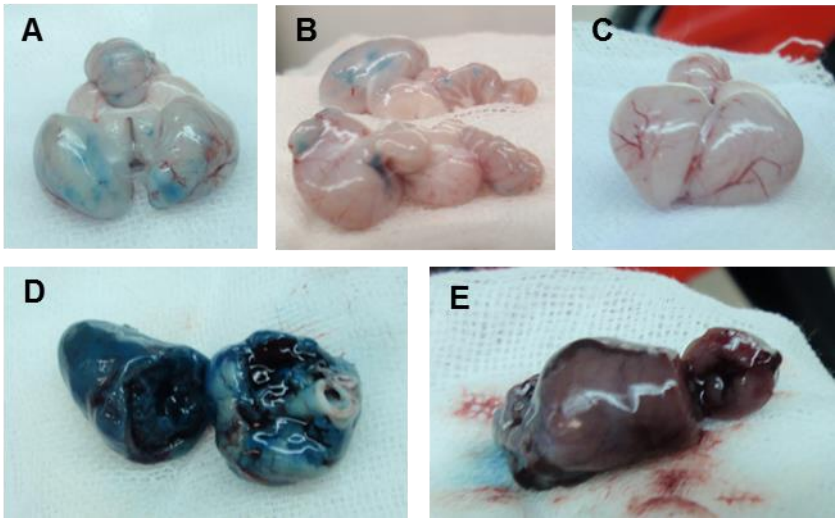

Fig. 6　Extravasation of Evans blue in the tissues of the chickens.

Four days after infection, Evans blue was intravenously injected into the chickens, and tissues were collected 3 h later.　Photographs show brains of the chickens inoculated with Ty/Italy (A and B) or Ck/Ibaraki (C) and hearts of the chickens inoculated with Ty/Italy (D) or Ck/Ibaraki (E).　Asterisk in B indicates stained choroid plexus.

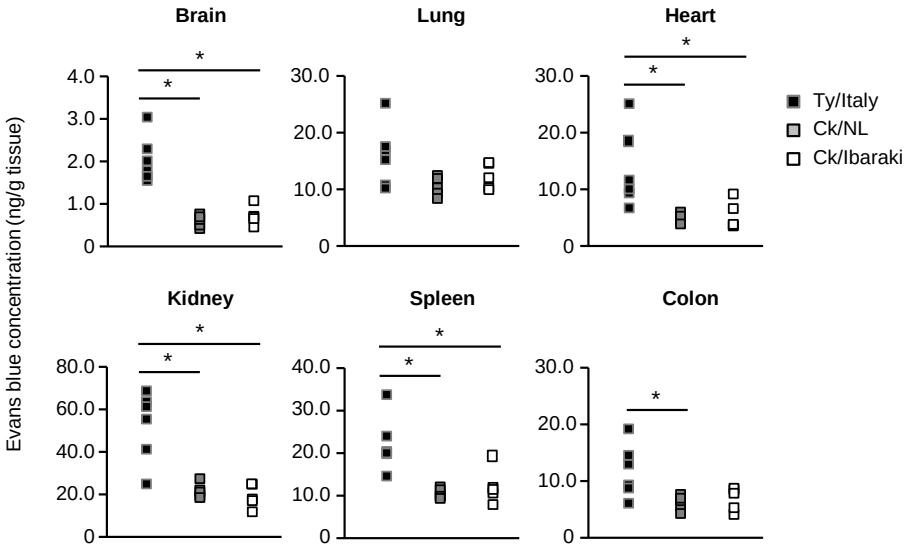

Fig. 7　Evans blue concentrations in the tissues of individual chickens.

Values from the chickens inoculated with Ty/Italy (n = 6), Ck/NL (n = 5), and Ck/Ibaraki (n = 5) are shown with closed squares, gray squares, and open squares, respectively. Asterisks indicate significant differences between 2 groups ($P<0.05$).

. Of note, focal necrosis of neurons, necrosis and cellular filtration into ependymal cell layers, swollen choroid plexus, thrombus formation, and hemorrhage were observed (Figs. 8a–8c). For comparison, no extravasation of Evans blue dye was found in the Ck/Ibaraki-infected or normal chickens (Figs. 6C, 6E, and Fig. 7).

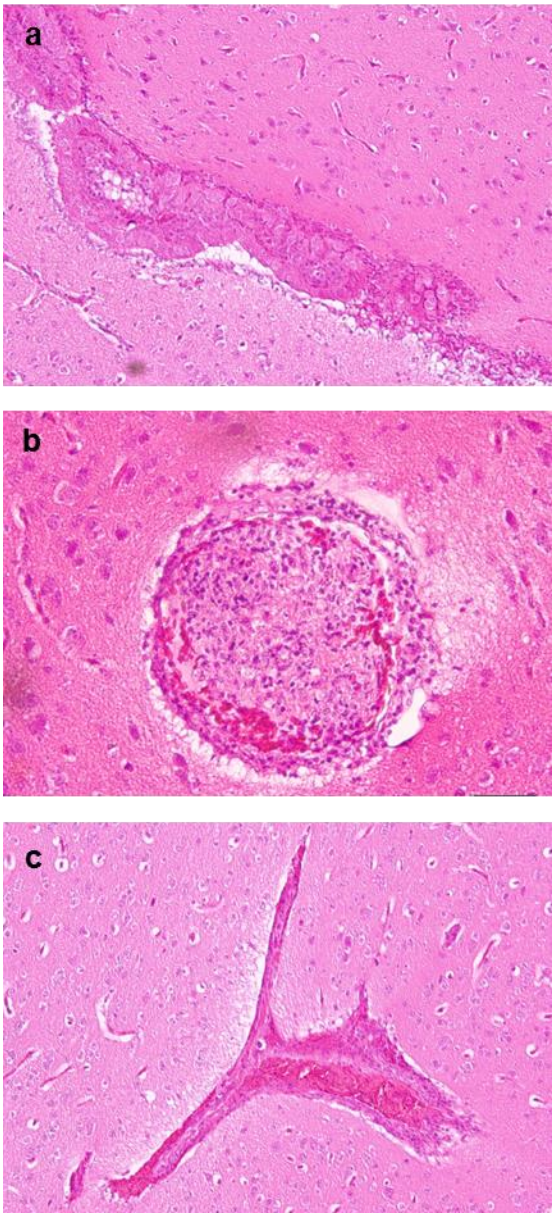

Fig. 8 Histopathological findings in the brain of the dead chicken infected with Ty/Italy.
After Evans blue inoculation, stained brains of the chickens infected with Ty/Italy was subjected to histopathological analysis. Photographs show swollen choroid plexus (a), thrombus (b), and hemorrhage (c) in the brain of the chicken. Original magnification, ×100: a, c; ×200: b

Discussion

Rapid and extensive proliferation of viruses was followed by rapid pro-inflammatory and antiviral cytokine response in tissues of the chickens inoculated with Ty/Italy, with severe capillary leakage in multiple organs during the acute phase of infection. Only moderate viral proliferation and cytokine response were observed in the chickens inoculated with Ck/NL, and no capillary leakage was observed in any of the tissues examined. Local and asymptomatic infection with a LPAIV, Ck/Ibaraki did not cause significant cytokine response or capillary leakage. These results indicate that differences in the pathogenicity of Ty/Italy, Ck/NL, and Ck/Ibaraki depend on the extent of the cytokine response, which was proportional to the proliferation of each virus in chickens. Others also demonstrated the relationship between the pathogenicity of HPAIVs and cytokine responses in chickens [31, 45, 55].

Gross lesions implying vascular damages including edema and hemorrhage rather than nocroses, have been found in the tissues of the chickens died of acute infection with HPAIVs [35, 56]. In the present study, extravasation of Evans blue, indicating capillary leakage, was correlated with the extent of cytokine response in each tissue of the chickens infected with Ty/Italy. The most significant Evans blue extravasation was found in the brains of the chickens infected with Ty/Italy, accompanied by

thrombus formation and hemorrhage. Pro-inflammatory cytokines cause a decrease in tight junction proteins between endothelial cells, leading to hyper vascular permeability [15, 52, 63]. In addition, IL-1β, IL-6, and TNF-α are known to activate coagulation systems in infections, trauma, inflammation, and cancer [27]. Muramoto *et al.* [32] demonstrated that H5N1 HPAIV infection caused activation of coagulation factor, tissue factor (TF) in chickens. TF is constitutively expressed in vascular smooth muscle cells and fibroblasts, and produced in macrophages and endothelial cells following stimulation with inflammatory cytokines [14]. These findings strongly support that edema of multiple organs and coagulopathy in HPAIV infection should be led by an excessive cytokine response, resulting in cardiovascular abnormality and consequent multiple organ failure in this chicken model.

Among the cytokines examined in the present study, IL-6 response was most significant in the chickens infected with HPAIVs. In the brain of the chickens infected with Ty/Italy, mRNA of IL-6 was detected in the microglia. Similar to bone-marrow-derived macrophages in peripheral tissues, microglia serves as the first defenders against infectious agents or injury-related products in the central nervous system [42]. These findings indicate that macrophages including microglia are stimulated by virus proliferation to release cytokines, especially IL-6, and play a key role in driving the excessive cytokine production in chickens infected with HPAIVs.

In addition, non-immune cells must also contribute to the amplification of cytokine response in chickens infected with HPAIV, although the mechanisms of cytokine amplification have not been clarified in the present study. In other studies, it has been shown that IL-6 is produced by various types of cells including immune cells, keratinocytes, endothelial cells, fibroblasts, astrocytes, mesangial cells, and bone marrow stroma cells [1]. Nakajima *et al.* [34] recently demonstrated that monocytes/macrophages, alveolar epithelial cells, and endothelial cells were dominant producers of IL-6 in the lungs of the patient infected with H5N1 HPAIV by immunohistochemistry.

Excessive cytokine response and multiple organ failure followed by death of the host are found commonly in other viral and bacterial infections than highly pathogenic avian influenza [6, 10, 26, 43]. The present results, thus, should contribute to the understanding of the pathogenesis of infections, leading the development of therapeutic drugs against those.

Brief summary

Highly pathogenic avian influenza viruses (HPAIVs) cause lethal infection in chickens. Severe cases of HPAIV infection have been also found in mammals including humans. In both mammals and birds, the relationship between host cytokine response to the infection with HPAIVs and lethal outcome has not been well understood. In the present study, HPAIVs A/turkey/Italy/4580/1999 (H7N1) (Ty/Italy) and A/chicken/Netherlands/2586/2003 (H7N7) (Ck/NL) and a low pathogenic avian influenza virus (LPAIV), A/chicken/Ibaraki/1/2005 (H5N2) (Ck/Ibaraki) were intranasally inoculated into chickens. Ty/Italy replicated more extensively than Ck/NL in systemic tissues of the chickens, especially in the brain, and induced excessive mRNA expression of pro-inflammatory and antiviral cytokines (IFN-γ, IL-1β, IL-6, and IFN-α) in proportion to its proliferation. Using *in situ* hybridization, IL-6 mRNA was detected mainly in microglial nodules in the brains of the chickens infected with Ty/Italy. Capillary leakage assessed by Evans blue staining was observed in multiple organs, especially in the brains of the chickens infected with Ty/Italy, and was not observed in those infected with Ck/NL. In contrast, LPAIV caused only local infection in the chickens, without apparent mRNA expression of cytokines and capillary leakage in any tissue of the chickens. The present results indicate that an excessive cytokine response

is induced by rapid and extensive proliferation of HPAIVs and causes fatal multiple organ failure in chickens.

Chapter II

The role of IL-6 in the pathogenesis of highly pathogenic avian influenza virus infection in chickens

Introduction

The infection with HPAIVs results in nearly 100% mortality in chickens. Some HPAIVs have been shown to be highly pathogenic in experimental animals such as mice, ferrets, and monkeys [4, 5, 26, 64]. H5N1 HPAIV infections in humans have been also found in Asian countries, causing a serious public health concern [10, 26, 62].

In Chapter I, it was demonstrated that extensive proliferation of HPAIV and excessive mRNA expression of cytokines, especially IL-6, were associated with severe vascular damages including multiple organ edema, hemorrhage, and coagulopathy in chickens. It is known that IL-6 is produced in the early stage of infection along with TNF-α and IL-1β, and stimulates the acute phase reaction and innate immune response. These inflammatory cytokines work synergistically, increasing vascular permeability, and mediating coagulation and febrile response in the hosts. IL-6 has also important roles as anti-inflammatory cytokine to control TNF-α and IL-1 levels [53, 60]. Marked

IL-6 response has been found in cases of influenza, viral hemorrhagic fever, and septic shock [5, 6, 26, 43]. However, it is not known how excessive IL-6 response affects the pathogenesis of these acute infectious diseases in the hosts.

In Chapter II, it is described that the role of IL-6 in the pathogenesis of HPAIV infection in chickens was investigated using recombinant chicken IL-6 (rchIL-6) and anti-rchIL-6 antibodies.

Materials and Methods

Cell lines

Chinese hamster ovary cells carrying chicken IL-6 gene (CHO6-2 cells) and HUC2-13 cells [29] were kindly provided by Dr. Haruo Matsuda (Hiroshima Biomedical Co. Ltd., Higashi Hiroshima, Japan). Both cells were cultured in Iscove's modified Dlubecco's medium (IMDM) (Life Technologies, California, U.S.A.) containing 5% fatal bovine serum (Thermo Scientific, Massachusetts, U.S.A.), 100 U/ml penicillin G (Meiji Seika, Tokyo, Japan), 0.1 mg/ml streptomycin (Meiji seika), and 8 μg/ml gentamicin (Merk, New Jersey, U.S.A.). CHO6-2 cells stably secret histidine-tagged rchIL-6 in the cultured supernatant. HUC2-13 cells are IL-6 responsive, and they were used for *in vitro* activity evaluation of rchIL-6 and anti-rchIL-6 antibodies.

Purification of rchIL-6

His-tagged rchIL-6 was purified from the CHO6-2 cell cultured supernatant by metal affinity using His Trap HP pre-packed column (GE Healthcare, Buckinghamshire, UK) and AKTA prime chromatography system (GE Healthcare). The supernatant was filtered by 0.45 μm membrane filter (Millipore, Massachusetts, U.S.A.) and was loaded

onto the column pre-equilibrated with binding buffer (20 mM phosphate buffer containing 20 mM imidazole and 500 mM NaCl) (pH 7.4). After washing with binding buffer, rchIL-6 was eluted with 20 mM phosphate buffer containing 500 mM imidazole and 500 mM NaCl (pH 7.4). The eluted fractions were dialyzed with Spectra/Por 1 Dialysis Membrane (Spectrum Laboratories, California, U.S.A.) in PBS containing 0.01% Tween20 (Nacalai tesque, Kyoto, Japan) at 4^{o}C overnight. The purity of rchIL-6 was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. The rchIL-6 protein was verified by Western blotting (WB) with mouse anti-rchIL-6 monoclonal antibody [39] provided by Dr. Haruo Matsuda. The concentration of rchIL-6 was determined by BCA protein assay Kit (Thermo Scientific) and image analysis of the intensity of Coomassie Brilliant Blue (CBB) stained bands after SDS-PAGE using Image J Software (National Institute of Health, Maryland, U.S.A.).

Immunization of rabbits with rchIL-6 and generation of hyper-immune serum

Two 12-week-old rabbits were intramuscularly immunized with 500 μg of rchIL-6 in Freund's incomplete adjuvant (Becton, Dickinson and Company Japan, Tokyo, Japan) two times at two weeks intervals. Booster injections of 500 μg of rchIL-6 were performed at 2 weeks later the second immunization. After 5‐6 days, immunized

serum was collected, and anti-rchIL-6 antibody titers and the concentrations of anti-rchIL-6 IgG were determined by Enzyme-Linked Immunosorbent Assay (ELISA) [21].

SDS-PAGE and WB analysis

SDS-PAGE and WB was performed by the methods of Laemmli [23] and Towbin *et al.* [61]. Samples were boiled with SDS loading buffer [0.1 M Tris-HCl (pH6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and bromophenol blue] for 5 min and they were separated by 7.5, 12, or 15% SDS-polyacrylamide gel electrophoresis. Separated proteins were then electrophoretically transferred to an Immobilon-P transfer membrane (Millipore). The membrane was treated with 5% non-fat milk, then treated with primary antibodies and treated with Horseradish peroxidase (HRP)-conjugated secondary antibodies. The membrane was developed with Immobilin Western Chemiluminescent HRP Substrate (Millipore). The bands were visualized with LumiVision PRO (AISIN SEIKI, Aichi, Japan).

Biological activity of rchIL-6 and anti-rchIL-6 antibodies in vitro

The activity of rchIL-6 and anti-rchIL-6 antibodies was evaluated as described previously [38] with some modifications. Briefly, HUC2-13 cells were cultured at a

concentration of 3×10^6 cells/ml in 6-well-culture plates in serum-free IMDM at 37 °C for 12 h. For the evaluation of rchIL-6 activity, the cells were treated with 0, 10, 100, and 1,000 ng/ml of rchIL-6. To examine the inhibition of IL-6 signaling by anti-rchIL-6 antibodies, cells were treated with 100 ng/ml of rchIL-6 pre-incubated with anti-rchIL-6 rabbit serum for 1 h. After 15 min incubation at 37 °C, cells were harvested and lysed with lysis buffer [0.1 M Tris-HCl (pH6.8), 4% SDS, 20% glycerol] containing Complete mini protease inhibitor (Roche Diagnostics, Mannheim, Germany) and PhosSTOP inhibitory cocktails (Roche Diagnostics). The equivalent 10 μg of proteins were separated on a 7.5% polyacrylamide gel and phosphorylated signal transducer and activator of transcription (STAT3) (p-STAT3) or STAT3 was detected with specific antibodies [anti-mouse p-STAT3 (Y705) antibodies, #9139 (Cell signaling Technology, Massachusetts, U.S.A.) and anti-mouse STAT3 antibodies, bs-1141R (Bioss, Massachusetts, U.S.A.)] by WB.

Animal experiments

In order to examine the effect of rchIL-6 on tissue vascular permeability, 0.4, 2, 10 μg of rchIL-6 or PBS was intravenously administered into 3 six-week-old chickens (Line-M) (Nisseiken, Yamanashi, Japan) after the treatment of Evans blue dye (Wako, Osaka, Japan), as described in Materials and Methods of Chapter I. After 3 h, brains,

lungs, hearts, spleens, kidneys, and colons were collected. Tissue samples were soaked in formamide (Sigma Aldrich Japan, Tokyo, Japan) to analyze the Evans blue extravasation, as described in Materials and Methods of Chapter I.

In order to assess the effect of anti-rchIL-6 antibodies to HPAIV infection in chickens, 1 ml of anti-rchIL-6 rabbit serum (3.1 mg of anti-rchIL-6 IgG equivalent), normal rabbit serum, or PBS was intravenously inoculated into 3 five-week old chickens (Bris brown) (Hokkaido Chuo Shukeijyo Corporation, Hokkaido, Japan), 1 h before intranasal inoculation with $10^{6.0}$ EID_{50} of Ty/Italy/99. As a control, 1 ml of anti-rchIL-6 rabbit serum was injected into 2 chickens without virus inculation. All chickens were observed for 10 days and brains, lungs, hearts, and spleens were collected from dead chickens for virus titration.

All animals were housed in self-contained units (Tokiwa Kagaku, Tokyo, Japan) in the BSL-3 facility of the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The institutional animal care and use committee of the Graduate School of Veterinary Medicine approved these animal experiments (approval numbers: 13-13050), and all experiments were performed under the guidance of the Institute for Laboratory Animal Research.

Results

Production and purification of rchIL-6

The rchIL-6 was purified from the supernatant of cultured CHO6-2 cells with nickel affinity columns. In silver staining of polyacrylamide gel and WB analysis, rchIL-6 was identified as an approximately 30 kDa band (Figs. 1A and 1B). In BCA assay, approximate 0.5 mg of rchIL-6 was yielded from 100 ml of the cell culture supernatant. Purified rchIL-6 contained FBS or CHO cell derived molecules which were found at the area of more than 50 kDa (Fig. 1A). From the analysis of band intensity of rchIL-6 in comparison with that of BSA standard protein in CBB staining, the purity of rchIL-6 was approximately 20%.

Biological activity of rchIL-6 in vitro

Binding of IL-6 to its receptor induces dimerization of signal transducer gp130, resulting in phosphorylation of STAT1 or STAT3 in the activation of the Jak/STAT pathway [16]. To examine biological activity of rchIL-6 *in vitro*, 3×10^6 cells/ml of HUC2-13 cells were treated with 0, 10, 100, and 1,000 ng/ml of rchIL-6 at 37 ^{o}C for 15 min. By WB analysis, p-STAT3 increased in a dose-dependent manner (Fig. 2), indicating that rchIL-6 derived from CHO6-2 cell has biological activity in HUC2-13

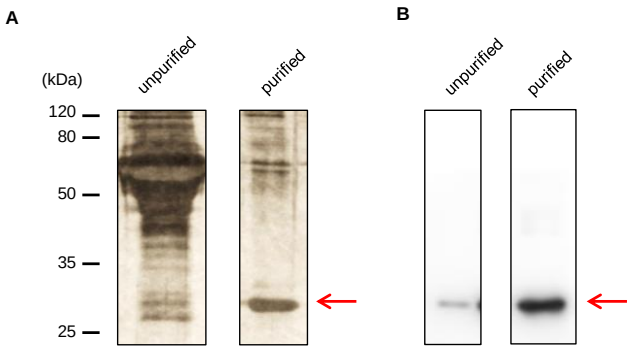

Fig. 1　Purity of rchIL-6 purified from CHO6-2 cell supernatants

The CHO6-2 cell culture supernatant (unpurified) and purified rchIL-6 (purified) was separated in 15% polyacrylamide gel and the molecules were detected by silver staining (A). The separated proteins were then electrophoretically transferred to an Immobilon-P membrane, and rchIL-6 was detected with mouse anti-rchIL-6 monoclonal antibody (B). Red arrows indicate the bands of rchIL-6.

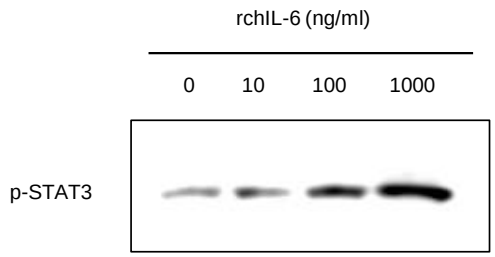

Fig. 2 Phosphorylation of STAT3 mediated by rchIL-6 in HUC2-13 cells

HUC2-13 cells were treated with 0, 10, 100, and 1,000 ng/ml of rchIL-6 at 37 ^{o}C for 15 min. After incubation, cells were lysed with lysis buffer and the equivalent 10 μg of proteins were separated on a 7.5% polyacrylamide gel. P-STAT was detected with anti-mouse p-STAT3 antibodies by WB.

cells.

Biological activities of anti-rchIL-6 antibodies

The ELISA titer of anti-rchIL-6 rabbit serum was 51,200. The concentration of anti-rchIL-6 IgG was 3.1 mg/ml. In order to examine whether anti-rchIL-6 antibodies inhibit intracellular signaling induced by rchIL-6, HUC2-13 cells were treated with 100 ng/ml of rchIL-6 pre-incubated with anti-rchIL-6 rabbit serum. Pre-incubation of rchIL-6 with anti-rchIL-6 rabbit serum decreased the levels of p-STAT3 in HUC2-13 cells, indicating that anti-rchIL-6 antibodies inhibited IL-6 signaling *in vitro* (Fig. 3).

Vascular permeability in tissues of the chickens treated with rchIL-6

To examine whether rchIL-6 increases tissue vascular permeability *in vivo*, Evans blue dye was intravenously inoculated into chickens. One hour later, 0.4, 2, 10 μg of rchIL-6 or PBS was intravenously administered. After 10 to 30 min of rchIL-6 administration, chickens appeared depressed, but they became normal within 1 h post administration. The concentrations of Evans blue in the kidneys of the chickens treated with 10 μg of rchIL-6 appeared higher than those of the chickens treated with 0.4 and 2 μg of rchIL-6 or PBS (Fig. 4). The absorbance values of the brains were too low to quantify the concentrations of Evans blue (data not shown).

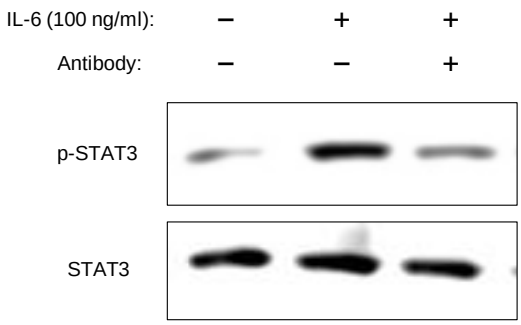

Fig. 3　Inhibition of IL-6 signaling in HUC2-13 cells by anti-rchIL-6 antibodies

HUC2-13 cells were treated with 100 ng of rchIL-6 pre-incubated with or without anti-rchIL-6 rabbit serum (3.1 μg of IgG equivalent) for 15 min.　After incubation, cells were lysed with lysis buffer. The equivalent 10 μg of proteins were separated on a 7.5% polyacrylamide gel and p-STAT3 or STAT3 was detected with specific antibodies by WB.

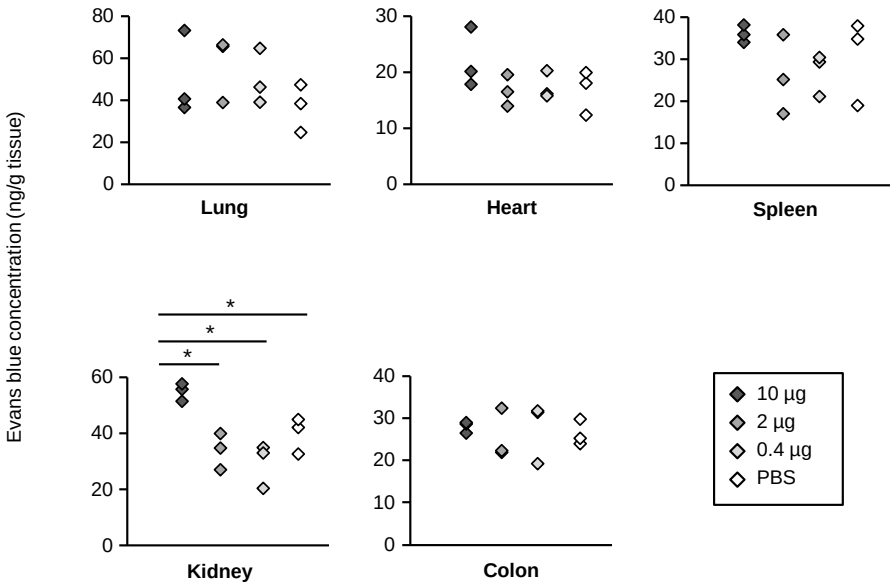

Fig. 4 Evans blue concentrations in the tissues of the chickens treated with rchIL-6

The Evans blue concentrations in the tissues of the chickens treated with 0.4, 2, 10 µg of rchIL-6 and PBS are analyzed. Asterisks indicate significant differences between 2 groups ($P<0.05$).

Anti-rchIL-6 antibodies treatment of chickens infected with HPAIV

In order to assess the effect of anti-rchIL-6 antibodies for HPAIV infection in chickens, 1 ml of anti-rchIL-6 rabbit serum was intravenously administrated into chickens at 1 h before the infection with $10^{6.0}$ EID_{50} of Ty/Italy. Depression was observed from 3 dpi in all chickens treated with anti-rchIL-6 rabbit serum, normal rabbit serum, or PBS, and all chickens died within 8 days (Fig. 5). No difference was found in the virus titers in the tissues of the chickens treated with anti-rchIL-6 rabbit serum and control birds (Fig. 6).

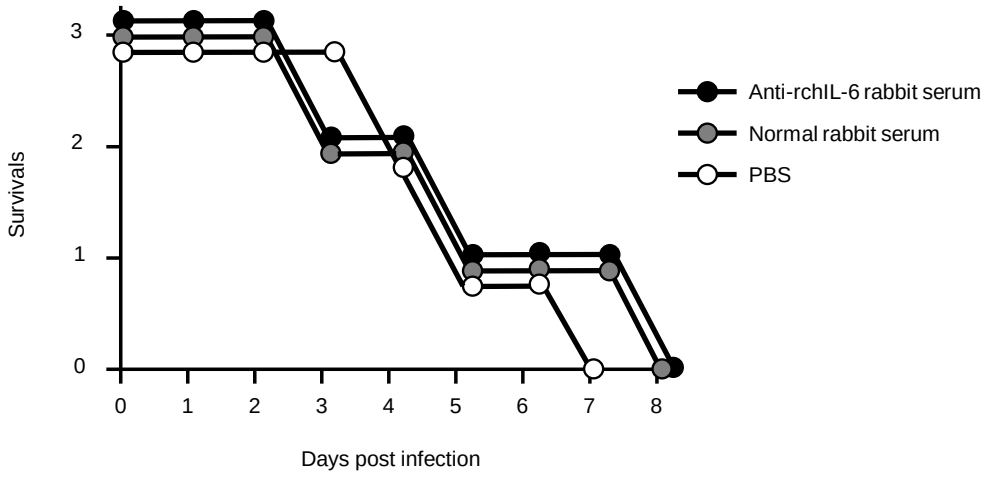

Fig. 5 Survival of the chickens inoculated with HPAIV intraveously pre-treated with rabbit sera or PBS

Chickens were treated with 1 ml of anti-rchIL-6 rabbit serum, normal rabbit serum, or PBS, and 1 h later, $10^{6.0}$ EID_{50} of Ty/Italy was intranasally inoculated.

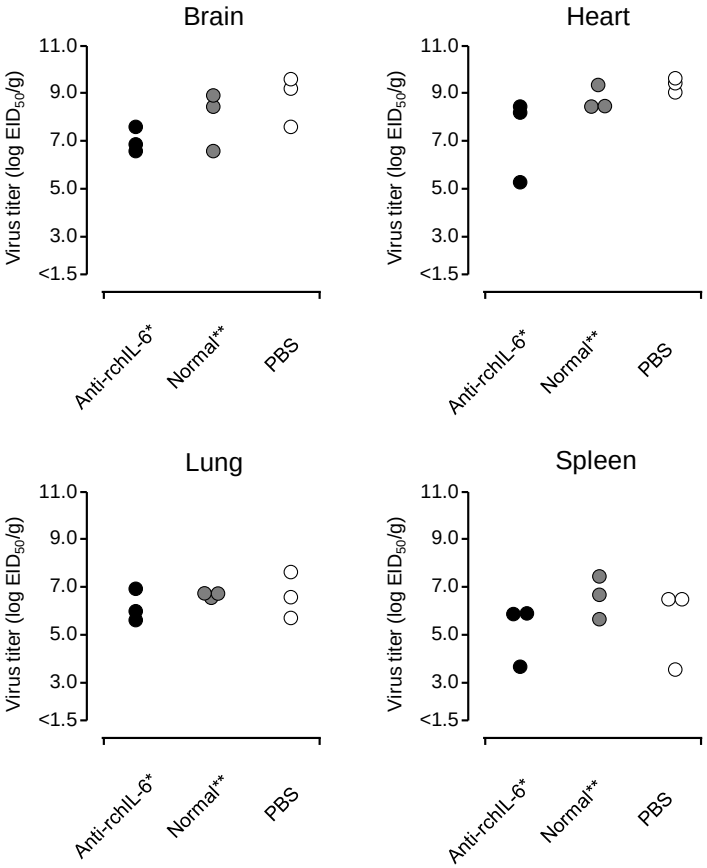

Fig. 6 Virus titers in the tissues of the chickens treated with rabbit sera or PBS before infection

Chickens were treated with 1ml of anti-rchIL-6 rabbit serum (*), normal rabbit serum (**), or PBS, and 1 h later, $10^{6.0}$ EID_{50} of Ty/Italy was intranasally inoculated. The tissues were collected from the dead chickens, and virus titers were determined and were presented as EID_{50}/g.

Discussion

It was demonstrated that the excessive response of cytokines, especially IL-6, to rapid proliferation of HPAIV led to fatal systemic capillary leakage in chickens, as shown in Chapter I. In order to define the role of IL-6 for the pathogenesis of highly pathogenic avian influenza, *in vivo* studies using rchIL-6 and anti-rchIL-6 antibodies were carried out.

Intravenous administration of rchIL-6 appeared to increase vascular permeability in the kidneys of the chickens, but did not cause the symptoms of HPAIV infection in chickens. It has been demonstrated that IL-6 is an important mediator to increase endothelial permeability *in vitro* [11, 28, 63]. Others showed that the lack of IL-6 decreased vascular permeability in the brains of mice in bacterial meningitis [44]. These findings indicate that IL-6 may contribute to systemic capillary leakage in chickens infected with HPAIVs. There are some different observations on the clearance of IL-6 from circulation. Cytokines are known to have very short half-life *in vivo* and cytokine-binding proteins such as soluble receptors or immunoadhesins are considered to control cytokine production in the circulation [3, 9, 13]. Castell *et al.* [9] demonstrated that exogenous recombinant human IL-6 (rhIL-6) bond to some plasma protein to be reversibly inactivated, and 80% of rhIL-6 was found in the liver 20 min

after intravenous injection in rats. Montero-Julian *et al.* [30] demonstrated that renal filtration was the major route of elimination of rhIL-6 in rats. These findings suggest that exogenous rchIL-6 might lose its biological activities by intrinsic clearance function in chickens.

Although anti-rchIL-6 antibodies inhibit biological activity of rchIL-6 in HUC2-13 cells, pre-treatment with the antibodies did not affect the survival of the chickens infected with HPAIV. It is required to determine IL-6 protein levels in the serum or tissues of the chickens infected with HPAVs and to apply appropriate dose of anti-rchIL-6 antibodies. It is known that antibodies derived from different species of animals are easily eliminated *in vivo*. Some compounds rather than antibodies, thus, may efficiently interfere with the activity of IL-6 to increase permeability of capillaries *in vivo*. Other pro-inflammatory cytokines and damage of endothelial cells resulting from extensive viral proliferation also may contribute to the systemic capillary leakage in chickens infected with HPAIVs. In particular, TNF-α and IL-1β are suggested as important host factors affecting pathogenesis in severe cases of influenza, hemorrhagic fever, and sepsis [6, 26, 43]. Qiang *et al*. [47] demonstrated that one of the endogenous molecules, cold-inducible RNA binding protein (CIRP) triggers inflammatory response, and anti-CIRP treated rats and mice survived from hemorrhage and sepsis. The intrinsic systems in endothelial cells, the roundabout axon guidance

receptor homolog 4-dependent Slit signaling which controls endothelial permeability, and the sphingosine-1-phosphate signaling which suppresses pro-inflammatory cytokine response and infiltration of immune cells, have been shown as possible targets for the treatment of multiple organ failure followed by excessive cytokine response [25, 59].

The present results suggest that excessive cytokine response is responsible for the pathogenesis of HPAIV infection in birds and mammals. HPAIV proliferation and excessive cytokine response, however, are observed almost simultaneously *in vivo*, as described in Chapter I. Combination of antiviral drugs and immunomodulators has been suggested as a preferential measure for the treatment of HPAIV infection [65], and such treatment may provide information to clarify the mechanism of the pathogenesis of highly pathogenic avian influenza and severe cases of influenza in humans.

Further investigations are ongoing to understand the role of excessive cytokine response in chickens infected with HPAIVs. The findings from such kind of studies must contribute to the development of therapeutic drugs for severe cases of influenza and other acute infectious diseases in animals and humans.

Brief Summary

Extensive virus proliferation and excessive mRNA expression of cytokines, especially IL-6, were associated with fatal systemic capillary leakage in chickens infected with highly pathogenic avian influenza virus (HPAIV). In order to elucidate how host cytokines, especially IL-6, affect the pathogenesis of HPAIV infection in chickens, recombinant chicken IL-6 (rchIL-6) and anti-rchIL-6 antibodies were used in the present studies.

In order to examine the effect of rchIL-6 on vascular permeability *in vivo*, Evans blue dye was intravenously injected into chickens and then 0.4, 2, 10 μg of rchIL-6 or PBS was intravenously administered. The concentration of Evans blue in the kidneys of the chickens treated with 10 μg of rchIL-6 appeared higher than those of the chickens treated with 0.4 or 2 μg of rchIL-6 or PBS, indicating that IL-6 may contribute to systemic capillary leakage in chickens infected with HPAIVs. However, injection of 10 μg of rchIL-6 did not induce the symptoms of highly pathogenic avian influenza, nor pre-treatment with anti-rchIL-6 antibodies affect the outcome of HPAIV infection in chickens. Not only cytokines including IL-6, TNF-α, and IL-1β but also the damage of endothelial cells caused by extensive viral proliferation may contribute to fatal capillary leakage in chickens.

Further studies are ongoing for better understanding of the relationship between excessive cytokine response and pathogenesis of HPAIV infection in chickens.

Conclusion

Highly pathogenic avian influenza viruses (HPAIVs) cause lethal systemic infection in chickens. Macroscopic pathological findings do not provide information about the cause of the death of the chickens infected with HPAIVs. Some H5N1 HPAIVs cause fatal disease in mammals including humans. Excessive cytokine response, called as “cytokine storm”, is found in both species, however, it has not been well explained how host cytokines affect the pathogenesis of HPAIV infections.

In the present study, two H7 HPAIVs, A/turkey/Italy/4580/1999 (H7N1) (Ty/Italy) and A/chicken/Netherlands/2586/2007 (H7N7) (Ck/NL), and a low pathogenic avian influenza virus (LPAIV), A/chicken/Ibaraki/1/2005 (H5N2) (Ck/Ibaraki) were inoculated into 4-week-old chickens. All of the chickens inoculated with Ty/Italy died within 4 days. Ty/Italy replicated extensively in systemic tissues of the chickens, inducing excessive mRNA expression of pro-inflammatory and antiviral cytokines (IFN-γ, IL-1β, IL-6, and IFN-α) in proportion to its proliferation. Capillary leakage assessed by Evans blue staining was observed in the multiple tissues of the chickens infected with Ty/Italy at 4 dpi. Half of the chickens inoculated with Ck/NL died in about a week. Virus titers and mRNA levels of cytokines in the tissues of the chickens inoculated with Ck/NL were about 100 times lower than those of the chickens

inoculated with Ty/Italy. Capillary leakage was not significant in any tissues of the chickens inoculated with Ck/NL at 4 dpi. In contrast, LPAIV, Ck/Ibaraki caused only a local infection in the chickens, without apparent cytokine expression and capillary leakage in any tissues of the chickens.

In order to elucidate the role of excessive response of cytokines, especially IL-6, in the pathogenesis of HPAIV infection in chickens, recombinant chicken IL-6 (rchIL-6) was prepared. Capillary permeability in the kidneys of the chickens pre-injected with Evans blue increased after administration of rchIL-6, indicating that IL-6 may contribute to systemic capillary leakage in chickens infected with HPAIVs. Anti-rchIL-6 antibodies which were generated by immunization of rchIL-6 to rabbits inhibited the biological activity of rchIL-6 *in vitro*, and pre-treatment with anti-rchIL-6 antibodies did not affect the outcome of the chickens infected with HPAIV.

The present results indicate that an excessive cytokine response induced by rapid and extensive proliferation of HPAIVs causes systemic capillary leakage, followed by multiple organ failure and sudden death in chickens. Not only excessive cytokines but also damages of the endothelial cells resulting from extensive viral proliferation may contribute to systemic capillary leakage in chickens infected with HPAIVs. Excessive cytokine response and multiple organ failure followed by the death of the hosts are found commonly in other viral and bacterial infections than highly pathogenic avian

influenza. The present results, thus, should contribute to the understanding of the pathogenesis of infections, leading the development of therapeutic drugs for the proper treatment of severe cases of influenza and other acute infections.

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和文要旨

鳥およびヒトを含む哺乳動物が高原性鳥インフルエンザウイルス (Highly pathogenic avian influenza virus; HPAIV) に感染し、急性経過を経て死亡する例では、宿主に過剰なサイトカイン応答が認められる。HPAIV の感染により急性経過で死亡したニワトリには、ほとんど肉眼病変を認めない。かかるニワトリの斃死は、ウイルスの感染増殖による組織の損傷よりも、増殖に対する宿主の過剰応答に起因するものと考えられる。本研究は、HPAIV のニワトリに対する病原性に、宿主のサイトカイン応答が寄与することを明らかにし、そのメカニズムの解明を試みたものである。

2 株の HPAIV、A/turky/Italy/4580/1999 (H7N1) (イタリア) 株、A/chicken/Netherlands/2586/2003 (H7N7) (オランダ) 株、および低病原性鳥インフルエンザウイルス A/chicken/Ibaraki/1/2005 (H5N2) (茨城) 株を 4 週令ニワトリに経鼻接種した。イタリア株を接種した 8 羽のニワトリは 4 日以内に全羽死亡した。脳、脾臓、肺において、ウイルスの急激な増殖と炎症性および抗ウイルス性サイトカイン (IFN-γ, IL-1β, IL-6 および IFN-α) mRNA の発現亢進を認めた。ウイルス接種 4 日後にエバンスブルーを静脈内に接種した結果、イタリア株感染ニワトリでは、全身の組織血管壁透過性が増していることがわかった。オランダ株を接種したニワトリは 7 日以内に半数が死亡した。組織におけるウイルス増殖およびサイトカイン mRNA 発現量は、イタリア株を接種したニワトリの約 1/100 で、感染 4 日後の組織血管壁透過性の増大は認められなかっ

た。

一方、茨城株を接種したニワトリは臨床症状を示すことなく生残し、ウイルスは一過性に呼吸器から回収された。いずれの組織においても顕著なサイトカイン応答、組織血管壁の損傷は認められなかった。

HPAIV 感染病態における過剰なサイトカイン、特に IL-6 の役割を明らかにするため、CHO 細胞発現組換えニワトリ IL-6 を、その培養上清から精製濃縮した。これをウサギに免疫し、抗ニワトリ IL-6 抗体を作出した。エバンスブルーを用いた試験によって、CHO 細胞発現組換えニワトリ IL-6 を静脈内に接種したニワトリの、組織血管壁透過性が増すことを明らかにした。ウサギ抗ニワトリ IL-6 抗体は *in vitro* でニワトリ IL-6 の生物活性を抑制したが、この抗体を静脈内に投与して 1 時間後に HPAIV を経鼻接種したニワトリの死亡を防ぐことはなかった。

以上より、HPAIV の急激な増殖に対する IL-6 をはじめとするサイトカインの過剰応答が、組織の血管障害を招き、その結果ニワトリは多臓器不全によって急死することが分かった。ただし、HPAIV に感染したニワトリにおける致死的な全身組織の血管障害は、IL-6 のみではなく他のサイトカインとの相乗作用、ならびにウイルスの感染、増殖による血管内皮細胞の損傷等によるものであると考えられる。過剰なサイトカイン応答は、インフルエンザだけでなく、さまざまな急性致死性感染症において認められる。ニワトリの HPAIV 感染病態における過剰なサイトカイン応答の役割について明らかにすることは、動物とヒトにおける急性致死性感染症の適切な治療法の開発につながるも

のと考え、実験を継続している。